THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 64:441–445, 1975

Vol. 64, No. 6
Printed in U.S.A.

HUMAN NECROTIZING VASCULITIS: IMMUNOGLOBULINS AND COMPLEMENT IN VESSEL WALLS OF CUTANEOUS LESIONS AND NORMAL SKIN

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Immunoglobulins and C3 were detected by immunofluorescence in the blood vessel walls of biopsies of clinically normal skin in patients with active necrotizing vasculitis. Of the 13 patients studied, 9 had C3 and 6 of these had IgM or IgA in biopsies of lesions of vasculitis. In adjacent clinically normal skin, 7 patients had C3 and 3 of these also had IgM or IgA. These findings support the hypothesis that immunoglobulins and complement are present in vessels of some patients prior to chemotaxis of polymorphonuclear leukocytes and the resulting inflammatory purpuric lesions so characteristic of necrotizing vasculitis.

Immunoglobulins and complement components may be found in the inflamed blood vessel walls of patients with a variety of forms of vasculitis [1]. Their presence has been used as presumptive evidence that antigen–antibody (immune) complexes may be of pathogenetic significance in the lesions of vasculitis. Inherent in such an hypothesis is the assumption that, first, circulating antigen–antibody complexes (with demonstrable immunoglobulins) are deposited, followed by local activation of complement and subsequent blood vessel wall damage. To date, however, all of the above events have been noted simultaneously in the same vessels and no time-course has been established for various steps in development of the vasculitis. This study describes the presence of immunoglobulins and complement in vessels from clinically normal skin of some patients‡ with active vasculitis.

MATERIALS AND METHODS

Patients. Of the 13 patients comprising this series, 11 presented with cutaneous palpable purpura, 1 with hemorrhagic bullae, and 1 with urticaria. The purpuric lesions varied in size from 1 mm to 10 mm, and frequently evolved to a necrotic center (Fig. 1).

Manuscript received November 11, 1974; in revised form January 23, 1975; accepted for publication January 30, 1975.

Supported in part by USPHS Grants AI 12028 and AI TI 00013, and by General Clinical Research Center Program of the Division of Research Resources (RR 51).

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‡The patients comprising this study are those being investigated in detail by the Vasculitis Study Group at the University of Colorado Medical Center. The patients and their clinical abnormalities will not be described in this manuscript.

Histopathologically, each lesion demonstrated endothelial cell swelling with destruction of the vessel wall, polymorphonuclear leukocytic infiltration, nuclear dust (leukocytoclasis), and hemorrhage (Fig. 2). Patients with these findings have been classified under such terms as necrotizing or leukocytoclastic vasculitis or allergic or hypersensitivity angiitis [2–4].

Immunofluorescence. Immunofluorescent studies were done on skin biopsies of lesions which had the same clinical appearance as and were within 1 to 5 cm of those lesions biopsied for routine histology. In addition, clinically normal skin within 1 to 5 cm was also biopsied for immunofluorescence. Normal skin was not biopsied for routine histology but was biopsied for electron microscopy from which thick sections were prepared and examined by light microscopy. Both specimens for fluorescence were immediately snap frozen in liquid nitrogen and stored at −70°C until sectioned. Frozen sections were embedded in O.C.T. Compound (Ames Co.), cut at 4 nm on an Ames cryostat, washed in 0.01 M phosphate-buffered (pH 7.2) saline for 10 min to remove excess O.C.T., and partially dried and overlaid with the respective fluorescein-labeled conjugate. Goat antihuman conjugates to IgG, IgM, IgA, C3, and fibrinogen were obtained from Hyland Laboratories, Los Angeles, California, and their monospecificity was confirmed by immunoelectrophoresis and double immunodiffusion in agar gel. Properdin was isolated by the Zymogen method [5] and the antibody to properdin was prepared in a horse and conjugated with fluorescein at the University of Colorado Medical Center. Specificity of the horse antiproperdin was confirmed by comparison with a rabbit antiproperdin donated by Dr. Irwin H. Lepow. The dilution of conjugate used was selected to give minimal background and maximum specific fluorescence on known positive control sections. The characteristics of the conjugates are given in Table I. After being washed in phosphate-buffered saline and mounted in glycerol, sections were examined with a Zeiss fluorescent microscope using a BG-12 primary filter and an 0-53 secondary filter.

The specificity of staining was determined by overlaying a section with unlabeled goat antihuman C3 followed by fluorescein-labeled antihuman C3 to determine whether specific blocking would result in an abolition of staining. For those tissues which stained with anti-C3 by

direct immunofluorescence, further complement component staining was performed using an indirect method. Tissue sections were first stained with unlabeled antihuman Clq, C4, C3, C5, or C3 proactivator (factor B). Antisera to Clq, C4, and C5 were prepared in goats in our laboratory using purified components. The anti-factor-B was from a rabbit and a gift of Dr. Otto Goetze at the Scripps Clinic, La Jolla, California. Controls, in addition to phosphate-buffered saline, included normal goat serum. After washing, sections stained with goat antihuman Clq, C4, C3, and C5 were stained with fluorescein-labeled rabbit antigoat IgG, while sections stained with rabbit anti-factor B were stained with fluorescein-conjugated anti-rabbit IgG. The reason for using an indirect method for the complement components was the resulting increased sensitivity as well as the unavailability of conjugated components.

FIG. 1. Characteristic lesions seen in patient 5. This man had numerous elevated purpuric lesions over his arms and trunk with confluence of individual lesions into large areas of necrosis on his legs.

Complement. Fresh serum was frozen at −70°C and total serum hemolytic complement was determined by the method of Mayer [6], while serum C3 was determined by radial immunodiffusion.

Other tests. Serum antinuclear antibody was determined by fluorescent staining of mouse liver nuclei, rheumatoid factor by latex agglutination, and the presence of hepatitis B antigen by electroimmunodiffusion. Cryoprotein levels were determined by methods previously described [7].

Patient controls. Cutaneous vessels from biopsies of 100 patients with a variety of skin diseases, including pemphigus, pemphigoid, erythema multiforme, and dermatitis herpetiformis, were specifically examined using the same fluorescein conjugates as above.

RESULTS

Pertinent characteristics of the 13 patients are given in Table II. In all patients the antinuclear antibody, rheumatoid factor, and hepatitis B antigen were negative. Six patients had abnormal amounts of serum cryoproteins but their presence did not correlate with the presence or absence of skin immunofluorescence.

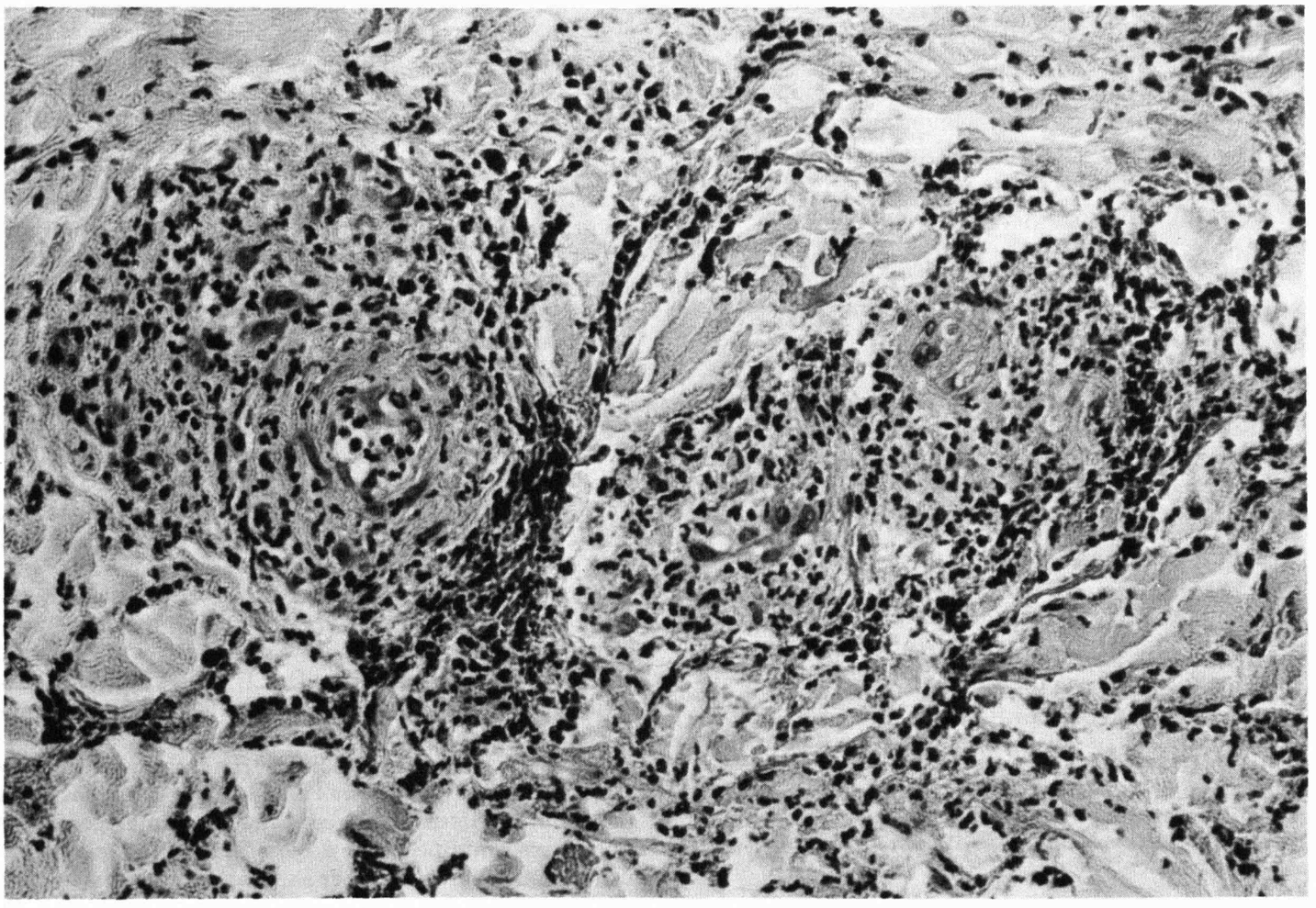

FIG. 2. Typical pathology seen in all patients in this study. This biopsy was from the lower leg of patient 4 and demonstrates a striking inflammatory reaction of the vessel walls. There is endothelial cell swelling, infiltration by polymorphonuclear leukocytes and leukocytoclasis (nuclear dust). The additional characteristic feature, hemorrhage, is not obvious in this view (× 88).

The results of the cutaneous immunofluorescence are summarized in Table III. All slides were read by a single experienced observer without knowledge of the patient or site of the sample. No IgG was detectable in any biopsy from either normal or involved skin. Positive staining by one or more conjugates was detected in the lesions of 10 of the 13 patients. The intensity of fluorescence was graded arbitrarily from 1 to 4+, with 1+ being weak but definite staining, and a 4+ indicating intense fluorescence. When found, positive staining was always in vessels of the mid-to-upper dermis, sometimes only those in the papillae. Staining appeared to be within or immediately surrounding the vessel wall and was finely granular in character (Fig. 3). In biopsies from involved skin, 9 of the 13 patients had vessel staining for C3, 4 for IgM, and 4 for IgA. No patient showed staining for IgG. Of the patients who were positive for C3, 4 were positive for C4, and 1 for Clq. The only patient whose vessels in a lesion had a bound immunoglobulin, but no C3, was #7 with IgA. Six patients had evidence of renal involvement, 2 of these confirmed by renal biopsy.

The most significant observation was positive staining in vessels from normal skin of 7 of the patients (Fig. 4). In some biopsies C3 staining in normal skin appeared considerably more intense than that from involved skin. In patient 9, staining for C3 and C4 in normal skin was positive while completely negative in a lesion. Fibrinogen seemed to be present in most vessels which showed positive staining by other conjugates.

The specificity of the staining was confirmed by blocking C3 staining with unlabeled anti-C3. No vessel staining was demonstrated in any of the controls except patients with systemic lupus erythematosus. Since no lesion stained with the conjugated anti-IgG, it was not felt that other negative controls were necessary to rule out nonspecific trapping of immunoglobulin and complement in the vessel wall.

Examination of thick sections of normal skin prepared for electron microscopy and stained with toluidine blue did not reveal any abnormalities.

TABLE I. *Characteristics of conjugates*

	Protein conc. (*mg/ml*)	Specific antibody conc. (*mg/ml*)	Fluorescein protein (*molar ratio*)	Protein-bound FITC conc. (*mg/ml*)	Dilution for use
Anti-IgG	21.2	1.3	2.4	125	1:20
Anti-IgA	21.9	2.4	2.5	132	1:10
Anti-IgM	20.0	1.9	2.6	126	1:2
Antifibrinogen	29.0	3.0	2.2		1:5
Anti-C3 (B1c/B1a)	12.3	2.0	3.4	102	1:20

DISCUSSION

This study demonstrates the presence of immunoglobulins and complement in the walls of cutaneous blood vessels of normal skin in some patients with active necrotizing vasculitis. The implication is obvious; it suggests that this form of vasculitis may be mediated by circulating antigen–antibody complexes, at least in some of the patients. We infer that the immunoglobulins represent antibodies bound to unidentified antigens. The fact that the vessels did not stain with all antisera used indicates that the immunoglobulins are specifically deposited in vessel walls. The only study similar to the present one was by Baart de la Faille-Kuyper et al [8] in which they demonstrated IgA and fragments of C3 but no Clq in normal skin ves-

TABLE II. *Vasculitis patient data*

	Cutaneous lesions	Proteinuria	Hematuria (*rbc/hpf*)	Renal biopsy	Hemolytic serum complement	Serum C3 (*mg/ml*)	Sedimentation rate (Westergren) (*mm/hr*)	Cryoglobulin (*mg/100 ml*)
Normal value		Neg	Neg		33–61	0.82–1.7	<15	<0.50 mg/100 ml
Patients								
1	Urticaria	Neg	Neg	nd	41	1.6	44	Neg
2	Palp purp	Neg	5–20	nd	35	1.3	17	nd
3	Palp purp	1+	3–5	GN	38	1.6	20	0.48
4	Palp purp	3+	5–10	GN	55	1.4	17	9.5
5	Palp purp	nd	5–20	nd	33	1.6	42	nd
6	Hemorrhagic bullae	Neg	Neg	nd	55	2.6	29	16.0
7	Palp purp	Neg	Neg	nd	48	1.3	18	0.83
8	Palp purp	Neg	Neg	nd	42	1.4	25	2.8
9	Palp purp	1+	Few	nd	49	2.1	21	0.65
10	Palp purp and urticaria	Neg	Neg	nd	34	1.6	16	Neg
11	Palp purp	Neg	Neg	nd	54	1.8	9	Neg
12	Palp purp	Neg	100–150	nd	47	2.7	nd	Neg
13	Palp purp	Neg	Neg	nd	45	2.1	28	6.9

Abbreviations: palp purp = palpable purpura; nd = not done; Hpf = high power field; GN = glomerulonephritis.

TABLE III. *Vasculitis immunofluorescence*

No.	Site of biopsy	Involved or uninvolved	IgA	IgM	Fibrin	Clq	C4	C3	C3pa	Properdin
1	Abdomen	Inv	—	—	—	nd	nd	—	nd	nd
	Abdomen	Uninv	—	—	—	nd	nd	—	nd	nd
2	Leg	Inv	—	—	—	nd	nd	—	nd	nd
	Leg	Uninv	—	—	—	nd	nd	—	nd	nd
3	Leg	Inv	1+	—	4+	—	—	1+	—	—
	Leg	Uninv	1+	—	3+	—	1+	3+	—	—
4	Leg	Inv	1+	1+	3+	nd	3+	3+	—	nd
	Leg	Uninv	—	—	—	—	2+	1+	—	—
5	Hand	Inv	—	—	2+	—	1+	1+	—	—
	Hand	Uninv	—	—	—	nd	nd	—	nd	nd
6	Leg	Inv	—	2+	—	nd	nd	1+	nd	nd
	Leg	Uninv	—	—	—	nd	nd	—	nd	nd
7	Abdomen	Inv	2+	—	2+	nd	nd	—	nd	nd
	Abdomen	Uninv	3+	1+	1+	nd	nd	2+	nd	nd
8	Foot	Inv	—	—	4+	—	—	2+	—	nd
	Foot	Uninv	—	—	nd	nd	nd	—	nd	nd
9	Leg	Inv	—	—	nd	nd	nd	—	nd	nd
	Leg	Uninv	—	—	2+	—	2+	2+	—	—
10	Leg	Inv	—	1+	nd	nd	nd	4+	nd	nd
	Leg	Uninv	—	—	nd	nd	nd	3+	nd	nd
11	Leg	Inv	—	—	nd	—	1+	2+	—	—
	Leg	Uninv	—	—	nd	—	—	2+	—	—
12	Leg	Inv	3+	—	nd	nd	nd	1+	nd	nd
	Leg	Uninv	nd	nd	nd	nd	nd	nd	nd	nd
13	Leg	Inv	—	1+	—	2+	4+	2+	—	—
	Leg	Uninv	—	1+	—	1+	3+	3+	—	—

Generally, sections were not stained for complement components if C3 was negative. In some cases, insufficient tissue was available.

Abbreviations: + = relative degree of positivity; — = negative; nd = not done; C3pa = C3 proactivator.

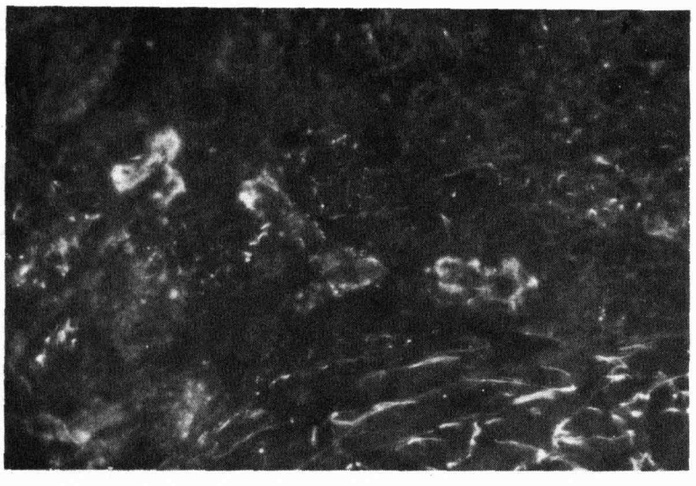

FIG. 3. Finely granular fluorescence of C3 in biopsy of skin lesion of patient 4. The linear fluorescent material is the autofluorescence of elastic fibers (× 70).

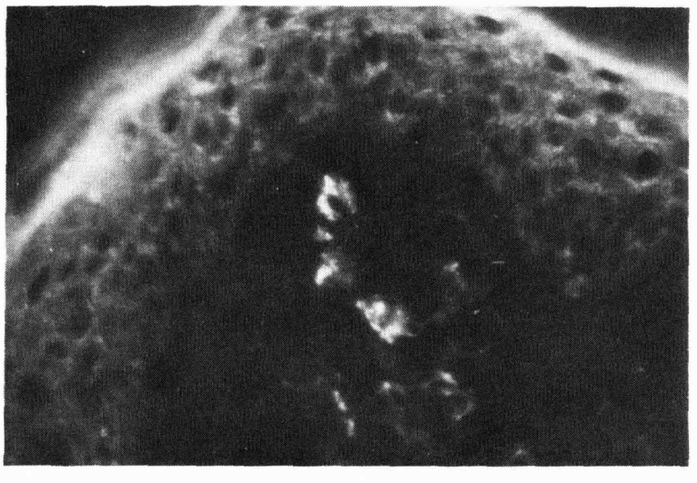

FIG. 4. C3 fluorescence in the vessels of a biopsy from normal skin of patient 3 (× 70).

sels of patients with Schönlein–Henoch purpura, suggesting to them activation of the alternate complement pathway.

It is not surprising that immunoglobulins and complement are not detected in lesions from some patients in this and other studies since Cochrane and Weigle [9] have shown experimentally that polymorphonuclear leukocytes will ingest and catabolize the antigen (and probably also the antibody) within 24 to 48 hr. Thus, it might reasonably be expected that the highest yield of positive immunoflurescence will be found in early lesions. Even more important from the pathogenetic standpoint is the finding of immunoglobulins or complement in vessels from clinically normal skin, but within the area where lesions have been forming. This implies that these vessels may soon be infiltrated by leukocytes resulting in a fully developed, palpable and purpuric lesion. It is also not too surprising that not all vessels in normal skin of the

patients demonstrate deposition of immunoglobulins since the clinical eruption is usually expressed as discrete lesions with intervening normal skin and does not involve all of a leg, for instance, diffusely. Reasons why the vasculitis would select specific vessels and spare others are obscure, but the observation that vasculitis usually begins and is most pronounced on the lower extremities may well be related to hydrostatic pressure and possibly "sludging." In rabbits the platelets presumably necessary for release of vasoactive amines have been demonstrated to clump in areas of vascular turbulence [10].

In 6 patients, complement components were detected and no immunoglobulin known to fix complement was observed. This is not surprising since C3 is the amplification step in the complement cascade and several hundred or thousand more C3 molecules may be bound in the vessel than Clq. Thus, since Clq is bound molecule for molecule to immunoglobulin, the amount of immunoglobulin bound may easily be below the detectable level of the fluorescent system used. In addition, C4 and C3, once activated, do not necessarily bind to the complex. These components may combine with vessel walls themselves much as they do to red blood cells. This may further explain the absence of immunoglobulins in the presence of complement.

The detection of Clq and C4 in some patients in the absence of detectable C3PA or properdin certainly indicates that the classical complement pathway is activated. The meaning of fibrin deposition is not clear, but may simply be related to the marked inflammation and vessel wall destruction, as we have found it in other inflammatory dermatoses such as erythema multiforme.

It was considered that the serum of these patients might contain an antibody for an antigen in the vessel walls to account for the staining seen by direct immunofluorescence. To examine this possibility, normal human skin was obtained from 4 different individuals, and cryostat sections overlain with serum dilutions of neat, 1:5 and 1:10 from each vasculitis patient. After washing, each section was stained with fluorescein-conjugated antibody to either IgG, IgM, or C3. In no instance was staining of normal vessels or other structures found.

The presence of immunoglobulins in the vessels of lesions of vasculitis has been appreciated for some years. Probably the first such demonstration was by Mellors and Ortega in 1956 [11] who found immunoglobulins in the arteries of a patient with periarteritis nodosa. Stringa and co-workers [12] studied 4 patients with a clinical disease similar to those in this report and found the vessels stained for IgG and C3 but not for IgM, albumin, or fibrinogen. Scott and Rowell [13] demonstrated globulin (type not specified) in the media or arterioles and the walls of capillaries in 3 patients having "necrotizing arteriolitis" but did not describe the clinical or pathologic characteristics in any detail. Paronetto [14] confirmed these findings in a single patient with hypersensitivity angiitis. The largest immunofluorescent study of necrotizing vasculitis is that of 26 patients reported by Schroeter et al [1]. They found IgG most frequently, but also IgM and C3, but no IgA. This is in striking contrast to our present study where no IgG was detectable in any patient. This might be explained if the IgG conjugate were weak or improperly made. However, the same conjugate regularly stained the dermal–epidermal zone of our lupus erythematosus biopsies used as controls for our conjugates.

These fluorescent antibody studies clearly demonstrating the presence of one or more immunoglobulins and complement in the vessels of clinically normal skin provide an additional observation to support the hypothesis that human leukocytoclastic vasculitis is an immune-complex disease.

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